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TITLE: Does Combination Immunotherapy with Human Monoclonal Antibodies against HER2 and CXCR4 Augment Breast Cancer Killing In Vitro and In Vivo

PRINCIPAL INVESTIGATOR: Wayne A. Marasco, M.D., Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

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Wayne A. Marasco, M.D., Ph.D.

E-Mail: wayne_marasco@dfci.harvard.edu

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Department of Chemical Engineering
University of California
Berkeley, CA 94720

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14. ABSTRACT
The chemokine receptor CXCR4 and its ligand CXCL12 (SDF1) have been proposedto regulate the directional migration and invasion ofbreast cancer cells to sites of metastasizes. The CXCR4 molecule could be a potential target to control breast cancer. Human epidermal growthfactor receptor-2 (HER2) overexpression contributes to tumor progression and metastasis. A humanized monoclonal antibody Herceptin(Trastuzumab) is currently in clinical use. Thus, both of CXCR4 and HER2 play important roles in breast cancer progress, the linkage betweenCXCR4 and HER2 has also been reported. HER2 upregulates the expression of CXCR4, which is required for HER2-mediated lung invasionand metastasis. Therefore, we aimed to assess the anti-tumor effects of combinational immunotherapy by targeting both CXCR4 and HER2 invitro and in a nude mouse breast cancer model. We have produced enough antibodies for the entire study, and established the CXCR4expressing cell lines for both in vitro and in vivo studies. We have evaluated the effects of anti- CXCR4 Mabs in combination of Herceptin oralone on inhibition of chemotaxis, invasion and proliferation on breast cancer cells. We also tested the function of two anti-CXCR4 Mabs inanimal model. To data, we have not been able to demonstrate anti-tumor activities of our anti-CXCR4 MAbs in vitro or in vivo which webelieve are, at least in part, due to technical problems with our assays and low expression of CXCR4 on the tumor cells used for in vivo assays.We are continuing to address these issues so we can answer the questions we have proposed in the future.

15. SUBJECT TERMS
Antibody, immunotherapy, Her2, CXCR4

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Introduction:

The chemokine receptor CXCR4 and its ligand CXCL12 (SDF1α) have been proposed to regulate the directional migration and invasion of breast cancer cells to sites of metastasizes (1). Inhibiting the interactions of CXCL12/CXCR4 either by antibodies against CXCR4 or small molecule antagonists impairs breast cancer metastasis in a mouse model. In addition to its role in breast cancer metastasis, an essential role of CXCR4 in breast cancer growth has been proposed by several studies. The CXCL12-CXCR4 signaling pathway is required for the regulation of the growth and the survival of both primary breast cancer cells and invasive or micrometastatic tumor cells. Inhibiting CXCR4 with RNAi, or the specific antagonist, substantially delayed the growth of breast cancer cells in SCID mice (2,3). Therefore the CXCR4 molecule could be a potential target to control breast tumor metastasis as well as growth.

Human epidermal growth factor receptor-2 (HER2), which is overexpressed in about 30% of all breast cancers, has been a target for antibody-based therapy for advanced breast cancer. A humanized monoclonal antibody Herceptin (Trastuzumab) is currently in clinical use. Despite careful patient selection on the basis of ErbB2 expression, only a minority of patients respond to trastuzumab monotherapy (4).

A study recently showed that HER2 upregulates the expression of CXCR4 by inhibiting CXCR4 degradation, which is required for HER2-mediated lung invasion and metastasis. A significant correlation between HER2 and CXCR4 expression was observed in human breast tumor tissues. Similar to HER2, CXCR4 expression correlated with a poor overall survival rate in patients with breast cancer (5).

The linkage between CXCR4 and HER2, both of which play important roles in breast cancer progress, provides the foundation for examining the anti-tumor effects of combinational immunotherapy by targeting both CXCR4 and HER2. Therefore in this grant we have proposed to assess the effect of combination treatment with human anti-CXCR4 Mabs we have identified and anti-HER2 antibody Herceptin on tumor growth and tumor metastasis in breast xenograft models.

Body:

We originally proposed to conduct in vitro and in vivo studies to determine if the combinational use of neutralizing human CXCR4 monoclonal antibodies (Mabs) with human anti-HER2 Mab (Herceptin) could act synergistically to treat breast cancer. However, in the first year we have encountered technical difficulties with the *in vitro* assays that would be used to identify the neutralizing CXCR4 Mab from a whole set of human CXCR4 Mabs generated by our lab.

In the original proposal, two major tasks were outlined. One of these, the *in vitro* experiment, it was divided into 5 aims :

1. Produce human anti-CXCR4 antibodies from stable CHO cell lines (Month 1-2).
2. Establish HER2 and luciferase stable expressing MDA-MB-231 breast cell line (Month 1-2).

3. Perform FACS analysis to evaluate down-regulation of CXCR4 expression with a series of different antibody treatments (Months 3).
4. Perform in vitro chemotaxis and invasion assays to evaluate whether synergistic inhibitory effects of antibodies against CXCR4 and HER2 are seen on the migration and invasion activity of breast cancer cells (Months 4-5).
5. Determine if the combination treatment cells with human anti-CXCR4 and Her2 Mabs will be more potent than a single agent in inhibiting breast cancer cell proliferation in vitro (Months 6).

In the first year, we have finished aims #1 and aim #2:

Aim #1, we have produced enough antibodies against CXCR4 for in vitro assays and animal studies from CHO stable cell lines, they are Mab 33 and 48.

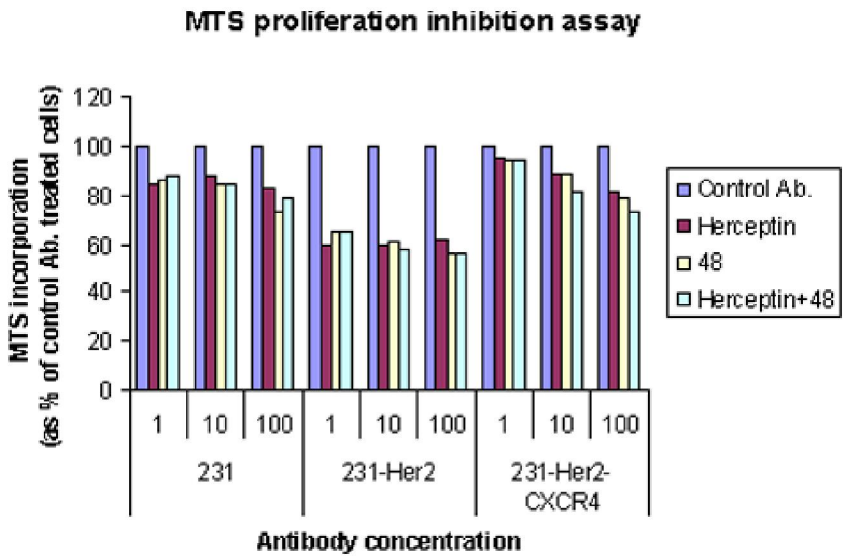
Aim #2, we have established Her2 and luciferase stable expressing MDA-MB-231 breast cell line by transducing the cells with Her2 and Luciferase expressing retroviral vectors. We also have established MDA-MB-231 cell line which expresses high level of Her2 and CXCR4. A luciferase expressing derivative cell line of it was also established.

What we have finished and the difficulties we have encountered in aims #3, 4 and #5 in the past year are listed as follows:

1 - Aim #3, we could not detect CXCR4 Mabs induced down-regulation of CXCR4 expression on breast cancer cell MDA-MB-231 and a few other breast cancer cell lines. We think the main reason for this might be that the baseline expression of CXCR4 on these breast cancer cells is low or even under the detection level of FACS analysis. Therefore we established a few high CXCR4-expression breast cancer cell lines including MDA-MB-231-CXCR4 and MCF7-CXCR4 to see if these cell lines could facilitate this assay to eventually provide us a definitive answer. However, we still could not see down-regulation of CXCR4 expression by CXCR4-Mabs on these CXCR4 high expression cell lines. Based on these data, we think this might not be a suitable assay to evaluate the function of CXCR4 Mabs.

2 - Aim #4, the in *vitro* chemotaxis and invasion assay. For the same reason as we mentioned above the baseline level of chemotactic and invasive activities of breast cancer cell, MDA-MB-231 (and a few others we have tried), are too low to be able to provide a sensitive screening assay for determining the neutralizing activity of CXCR4 Mabs. Therefore, we expected that high-CXCR4 expression on breast cancer cells could increase the sensitivity of this assay. Surprisingly, our experimental data showed, under various experimental conditions, these high-CXCR4 expressing sub-lines did not show increased chemotactic activity to SDF1α, while their ability of haptotaxis (haptotaxis involves the control of cell migration from a less adherent to a more adherent surface) to fibronectin was decreased dramatically. Although we do not have an explanation for this phenomenon, we believe there must be an unknown mechanism involving in chemotaxis, haptotaxis and invasion activities of these cells. In the future, we will continue to

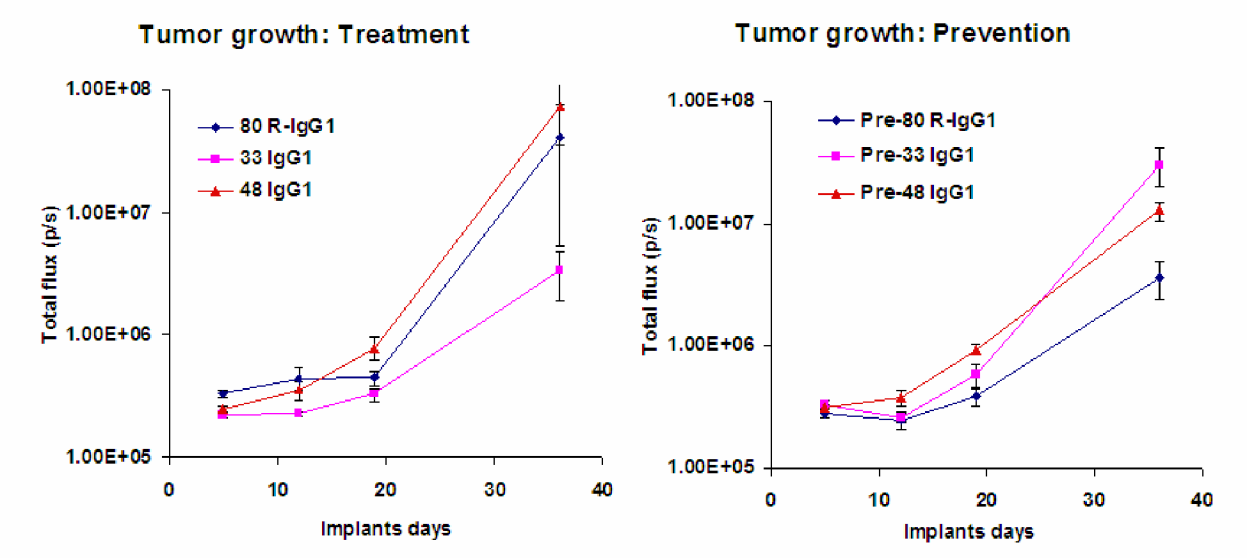
pursue a better understanding of what we have observed during these experiments and a better in vitro assay system to evaluate the function of our CXCR4 Abs.



3 - Aim #5, because of the technical difficulties we have had on aims # 3 and #4. This aim has been unavoidably delayed. However, we have tried one leading CXCR4 Mab 48, which has the highest binding activity to CXCR4, to see if combination treatment cells with it and Her2 Mab will be more potent than a single agent in inhibiting breast cancer cell proliferation. The result is shown on the left. The CXCR4 antibody 48 or Herceptin were shown to inhibit cell proliferation of MBA-MD-231-Her2 cells by about 40% at concentrations as low as 1 μg/ml. In contrast, significant inhibition of the Herceptin and 48 on parental MBA-MD-231 cells and MBA-MD-231-Her2-CXCR4 cells were not observed. The levels of growth inhibition mediated by Herceptin on parental cells and Her2 high expression cells are consistent with the results of another study (6). We did not found synergistic or additive proliferation inhibition effect of the combination of 48 and Herceptin antibodies in this assay.

The other task we have originally proposed is *in vivo* animal study to evaluate if the combined use of human Mabs against HER2 and CXCR4 can synergistically inhibit growth of xenografts, lung metastases and prolong overall survival. We have completed a pilot study with one CXCR4 Mab 48 in MDA-MB-231 breast cancer nude mouse model to test if it could reduce the metastasis of breast cancer cells. This study showed that 48 treatment reduced lung metastasis as compared to control group (1/5 versus 3/5 lung metastasis, respectively). We next did a more comprehensive and isotype Ab controlled animal study to test function of two anti-CXC4 Abs. Two experimental groups were set up. One is prevention group to evaluate if these two anti-CXCR4 have prevention effect in breast cancer metastasis: Abs were given intraperitoneally one day before injection of breast cancer cells; Second group is treatment group, antibody treatment began one week after cells injection. Each group was divided into 3 sub-groups: control antibody, anti-CXCR4 Ab 33 and 48. Each subgroup included 7 mice. Abs were given biweekly at 10mg/kg for 4 weeks. Lung metastasis of breast cancer was monitored twice a week using Xenogene imaging system. Neither anti-CXCR4 Abs showed a significant effect on prevention or treatment of lung metastasis of breast cancer compared with the control MAb (80R-IgG1) in this experiment (See figure below). This negative result may be explained by the control Ab was not a true negative control, it might have unknown or non-specific effect. We plan to use a different negative control

Ab in the future; we will also test other anti-CXCR4 Abs that recognize other epitopes on CXCR4 than the two antibodies we have tested.



Key research accomplishments:

- Produced sufficient human anti-CXCR4 Mabs for the entire study.
- Established cell lines which are necessary to perform both *in vitro* and *in vivo* animal studies.
- Evaluated down-regulation of CXCR4 expression by CXCR4 Mabs on breast cancer cell lines. We found this might not be a suitable assay to evaluate the function of CXCR4 Mabs.
- We found one CXCR4 Mab has the potential of cell growth inhibition activity *in vitro*.
- The function of two anti-CXCR4 Mabs in breast cancer animal model has been tested.

Reportable outcomes: A manuscript, abstract or presentation has not been resulted from this research. We have developed Herceptin, CXCR4 and Lucifease high expressing MBA-MD-231 cell lines.

Conclusions: Our anti-CXCR4 Mabs have demonstrated anti-proliferative effects on MBA-MD-231-Her2-CXCR4 cells that is equal to anti-Her2 Mab although additive or synergistic inhibition could not be demonstrated. However, it is not clear if anti-proliferative activity is directly responsible for the clearing of tumor cells *in vivo*. Indeed, immune mediated killing by antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) may be equally or more important. Though we have not completed all the planned studies, we will continue to pursue the important questions and determine if anti-CXCR4/Her2 immunotherapy is superior to either immunotherapy alone.

Appendices: None

Reference:

1. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001, 410: 50-56.
2. Smith MC, Luker KE, Garbow JR, Prior JL, Jackson E, Piwnica-Worms D, Luker GD. CXCR4 regulates growth of both primary and metastatic breast cancer. Cancer Res. 2004, 64(23): 8604-8612.
3. Lapteva N, Yang AG, Sanders DE, Strube RW, Chen SY. CXCR4 knockdown by small interfering RNA abrogates breast tumor growth in vivo. Cancer Gene Ther. 2005,12(1): 84-89.
4. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001 Mar 15;344(11):783-92.
5. Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M, Zhou X, Xia W, Hortobagyi GN, Yu D, Hung MC. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. Cancer Cell. 2004,6(5): 459-469.
6. du Manoir JM, Francia G, Man S, Mossoba M, Medin JA, Viloria-Petit A, Hicklin DJ, Emmenegger U, Kerbel RS. Strategies for delaying or treating in vivo acquired resistance to trastuzumab in human breast cancer xenografts. 2006, Clin Cancer Res. 12:904-16.